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Award Number: DAMD17-97-1-7046

TITLE: Cofactors that Mediate Facilitated Tubulin Folding as Novel Targets for Breast Cancer Chemotherapy

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REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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DTIC QUALITY INSPECTED 4

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Annual (15 Sep 98 - 14 Sep 99)

4. TITLE AND SUBTITLE
Cofactors that Mediate Facilitated Tubulin Folding as Novel Targets for Breast Cancer Chemotherapy

5. FUNDING NUMBERS
DAMD17-97-1-7046

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Microtubules are dynamic polymers that are essential to cell division as a major component of the mitotic spindle, and consist largely of two soluble proteins termed α- and β-tubulin. The biological activity of these proteins depends critically on their proper folding. This is a multi-step process, involving ATP-dependent interaction with cytosolic chaperonin, followed by an obligatory cascade of ATP-independent interactions with several protein cofactors. These proteins (termed cofactors A, B, C, D and E) function specifically in the α- and β-tubulin folding pathways. The goal of the project proposed in this application is to use the tools of contemporary molecular biology to search for compounds that act as specific inhibitors of one or more of these proteins. Because the synthesis of native tubulin is essential for cell division, reagents that specifically prevent the productive folding of tubulin are likely to be useful as novel chemotherapeutic agents for the treatment of breast cancer, either alone or in combination with existing drugs. Because their mode of action would target the de novo production of functional tubulin rather than the biochemical properties of tubulin per se, the development of such drugs would result in a new class of anti-tumor compounds.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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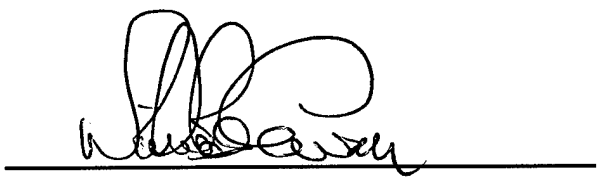

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INTRODUCTION

Virtually all eukaryotic cells contain microtubules. These are dynamic filamentous polymers that perform a variety of essential cellular functions, including the maintenance of cell shape, the intracellular transport of organelles, and cell division as part of the mitotic apparatus. Microtubules are assembled from subunits consisting of α- and β-tubulin, which together form a heterodimer. Although it was originally thought that the tubulin heterodimer was formed by self-assembly of newly synthesized α- and β-tubulin polypeptides (Detrich and Williams, 1978), our work has shown that heterodimer formation is a complicated process requiring interaction with many other components (molecular chaperones) (Tian et al., 1996, 1997; Lewis et al., 1996,1997). These chaperones include a multisubunit complex (termed chaperonin) which participates in the correct folding of tubulin molecules, as well as five other molecular chaperones that function in locking the α- and β-subunits together into the functional heterodimer. The fact that heterodimer formation follows a complex pathway offers an opportunity to interfere with this process at a number of points. Since de novo heterodimer production is essential to the formation of the mitotic spindle, disrupting the supply of tubulin heterodimers is likely to prevent cell division and may therefore be a useful interventional mode in cancer chemotherapy. The purpose of the research conducted under this award is to understand the tubulin folding pathway in detail, and to explore ways of interfering with the tubulin folding pathways and their regulation.

BODY

1 Purification of Milligram Quantities of Cofactor D

Five tubulin-specific chaperones termed cofactors A-E participate in the pathway leading to the formation of assembly-competent tubulin heterodimers (Tian et al., 1996,1997; Lewis et al., 1996,1997). To study the folding reaction biochemically, we require methods for the production of these proteins in recombinant host/vector systems, since purification of the cofactors from tissue sources is laborious, time-consuming, and yields too little material to be useful for biochemical analysis. We successfully expressed cofactors A, B, C and E in either E. coli cells or in insect Sf21 cells; however, we were not successful in producing biologically active cofactor D in either of these systems. We therefore constructed a recombinant adenovirus engineered for the expression of cofactor D. This was done by co-transfecting cultured host 293 cells with adenoviral DNA containing a deletion rendering it inviable, together with a plasmid containing complementing adenoviral sequences and including a full-length cDNA encoding cofactor D driven by the cytomegalovirus (CMV) promoter. Sequences generated by recombination in vivo would then lead to the production of viable (i.e. infectious) viruses.

Viral plaques appearing several days after transfection were amplified and viral DNA prepared. Such preparations were then assayed by PCR to determine whether the viruses indeed contained cofactor D-encoding sequences. Several such recombinant viruses were obtained (Fig. 1). These viruses were further amplified and used to infect cultured HeLa cells. total extracts of these cells were then examined for the expression of cofactor D. We found one isolate that did result in the overexpression of cofactor D in HeLa cells (Fig. 2).

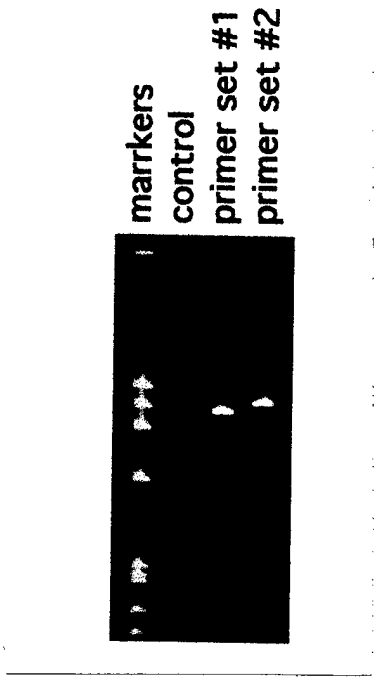

Fig. 1. PCR reaction products identifying recombinant adenoviruses for the expression of cofactor D. Three different primer sets are used on DNA from viral infected cells, the first identifying a sequence present only in nonrecombinant virus, the other two sets nested pairs amplifying recombinant cofactor D/viral sequence.

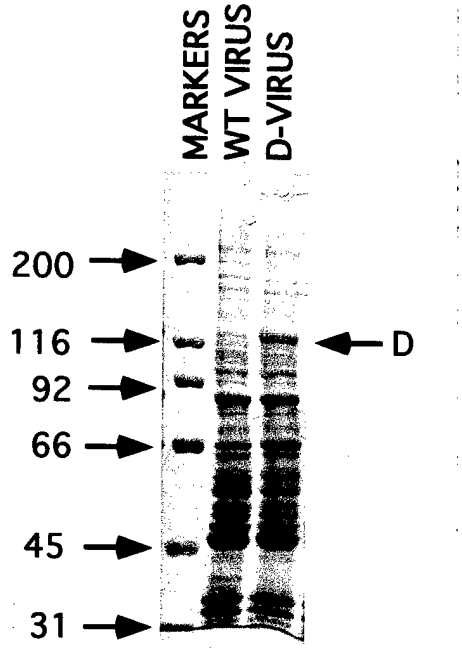

Fig. 2. The expression of cofactor D in HeLa cell infected with a recombinant cofactor D-encoding adenovirus. A Coomassie-stained SDS-PAGE gel. The positions of molecular mass markers (kD) and cofactor D are indicated by arrows.

2 Purification of Recombinant Cofactor D as a Co-complex with β-tubulin

We purified recombinant cofactor D from adenovirus infected HeLa cells using the chromatographic dimensions originally worked out for the purifiaction of cofactor D from tissue sources (Tian et al., 1996). We discovered that, upon analysis by SDS-PAGE, the

recombinant cofactor D copurified with β-tubulin as a cofactor D-β-tubulin complex (Fig. 3). We infer that overexpression of cofactor D in cultured cells results in the disruption of the cell's heterodimers, such that β-tubulin is sequestered as a cofactor D/β-tubulin complex (see below).

Although the complex purified from adenovirus infected HeLa cells was not the originally intended product, we found it to be useful in establishing the stoichiometry of this important intermediate in the overall tubulin folding pathway. Analytical ultracentrifugation showed the complex to contain one molecule each of cofactor D and β-tubulin. Experiments are currently under way to determine whether biologically active cofactor D can be obtained by the in vitro dissociation of the cofactorD/β-tubulin complex.

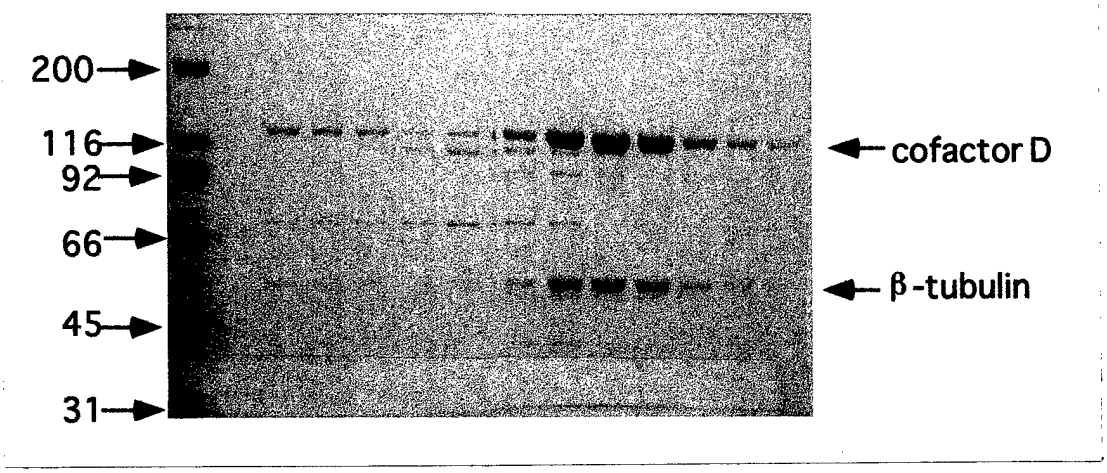

Fig. 3. Cofactor D expressed in adenovirus infected HeLa cells copurifies with β–tubulin. A semipurifed fraction containing cofactor D was fractionated on a gel filtration column, and analyzed by SDS-PAGE. The positions of cofactor D, β-tubulin and molecular size markers (kD) are indicated by arrows.

3 Proteolytic Digestion of Tubulin and its Effect on C-cpn-mediated Tubulin Folding.

With the goal in mind of identifying inhibitors (task 3), we did experiments to see which part of the α– and β–tubulin polypeptides interact with the folding machinery. To do this, we treated purified brain tubulin with subtilisin, which is known to remove the last 20 or so amino acids from each polypeptide chain and results in the formation of $\alpha_s\beta_s$ (Fig. 4a). Either native brain tubulin or $\alpha_s\beta_s$ was added to β-tubulin in vitro translation reactions done in the presence of ^{35}S-methionine, and the reaction products were resolved on non-denaturing gels (Fig. 4b). In reactions containing added unmodified tubulin, the yield of native labeled material is greatly enhanced relative to a control reaction done without added tubulin (compare tracks 1 and 2). In contrast, a parallel reaction done with added $\alpha_s\beta_s$ in place of unmodified tubulin resulted in a relatively poor yield of material migrating as the hybrid heterodimer $\alpha_s\beta$ (track 3). In either event, the production of heterodimers was inhibited by the addition of the slowly hydrolyzable GTP analog GTP-γ-S (tracks 3-6).

These data suggest that the carboxyterminal domains of α- and β-tubulin contribute to the overall GTP-dependent folding reaction. To test this hypothesis, we have synthesized peptides corresponding to the carboxyterminal domains. These will be assayed for their ability to inhibit α- and β-tubulin folding in vitro (task 7). In addition, we will pursue this approach further in the third year of the grant since it has shown promise. This should not interfere with accomplishing tasks 7 and 8 which are scheduled, provided we find inhibitors.

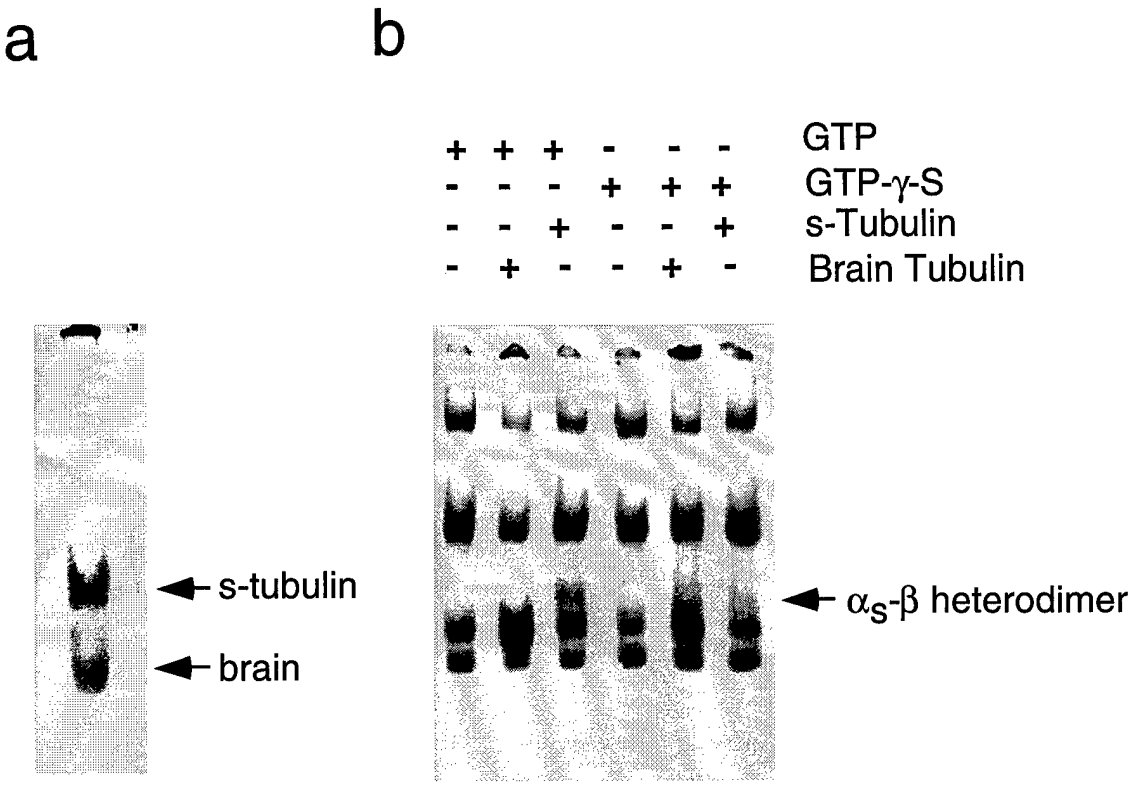

Fig. 4. Tubulin subfragments in folding reactions. S-tubulin was generated by cleavage of purified brain tubulin by subtilisin. Panel a shows a 1:1 mixture of s-tubulin and brain tubulin. Panel b shows the result of adding either s-tubulin or brain tubulin to in vitro translation reactions of β–tubulin.

4 Generation by PCR of fragments encoding portions of α- and β-tubulin.

We have begun task 2 by generating C-terminally truncated tubulins, to complement the experiment described in section 4. We used oligonucleotide primers to make βΔ9 and αΔ12, cDNAs encoding β-tubulin lacking its last 9 amino acids and α-tubulin missing its last 12 amino acids, respectively. We found that neither truncated tubulin would fold efficiently in vitro either by translation or by dilution from denaturant (Fig. 5). In addition, β–tubulin/cofactor D intermediates do not form with βΔ9, implying (as above) that the carboxyterminus is important for interaction with cofactors. We therefore hope to find that a peptide corresponding to this region will be an inhibitor. This approach will be pursued further in the third year of the grant, since it has shown promise

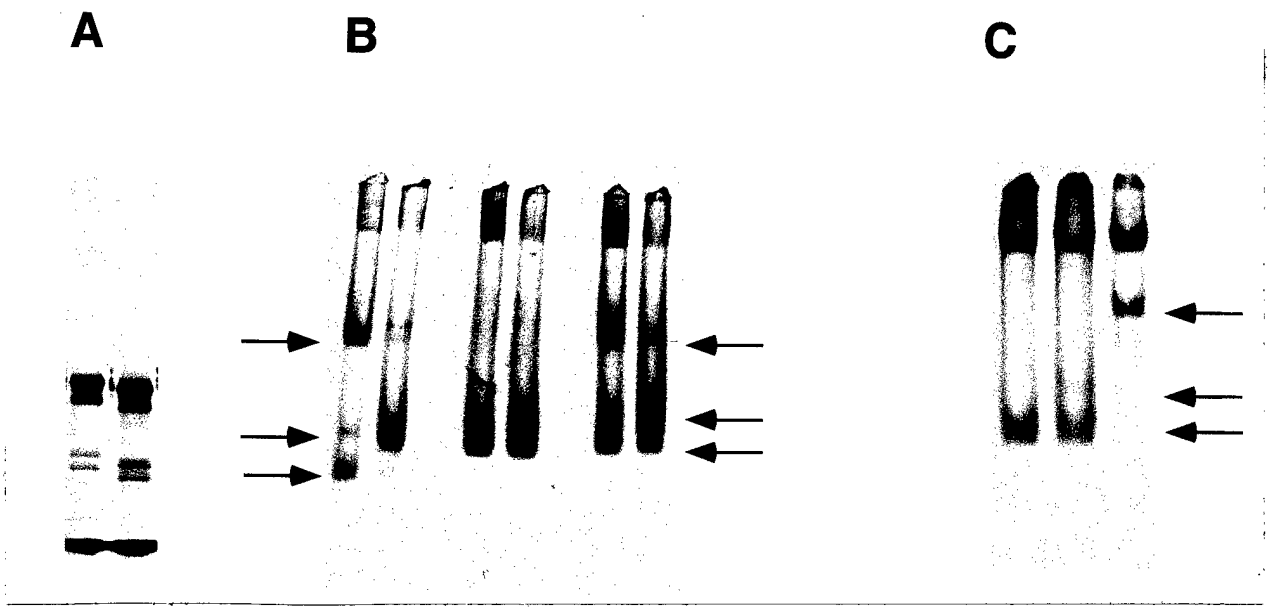

Fig. 5. βΔ9 and αΔ12 are not efficiently folded in vitro. Panel A. A Coomassie-stained SDS gel showing radiolabeled βΔ9 (left) and β-his (i.e. full-length tubulin with a carboxy-terminal his tag) (right). These proteins were used in in vitro translation reactions, and the products resolved on a non-denaturing gel, shown in panel B: left; β-tubulin, center; βΔ9, right; β-his. The first track in each set is a tubulin translation reaction without additions; the second track is the same reaction with added purified unlabeled tubulin to drive the reaction to completion. Arrows at left show, from top to bottom, the positions of cofactor D/β-tubulin complex, tubulin heterodimer and cofactor A/β-tubulin complex. Arrows at right show the positions of the same species for βΔ9 and β-his, which migrate more slowly on native gels due to charge differences. Panel C shows the folding of αΔ12 radiolabeled and denatured in 7M urea and presented to chaperonin plus cofactor B (lane 1), cofactors B, C, D and E and unlabeled tubulin (lane 2) or plus cofactors D and E and unlabeled tubulin (lane3). Chaperonin/ αΔ12 complexes migrate near the origin; arrows show the expected positions of cofactors Dand E/ αΔ12/β-tubulin complexes, αΔ12/β-tubulin heterodimer and cofactor B/ αΔ12 complexes respectively. Note that both truncated tubulins form little or no heterodimer.

5 Effects of cofactors in vivo. Preliminary to accomplishing task 8 (testing of inhibition of cofactors in cultured cells), we have constructed plasmids engineered for the expression of GFP-cofactor fusion proteins in mammalian cell. This will allow monitoring of the effects of inhibitors on cofactors, as well as titrating out inhibitors by overexpression of cofactors. In preliminary experiments we found that overexpression of GFP-cofactor D and GFP-cofactor E, but not GFP-cofactor C, was lethal in cultured cells, causing the complete loss of tubulin and microtubules (Fig. 6). This result supports the hypothesis on which the entire proposal was based, namely that interference with cofactor function will be lethal to cancer cells.

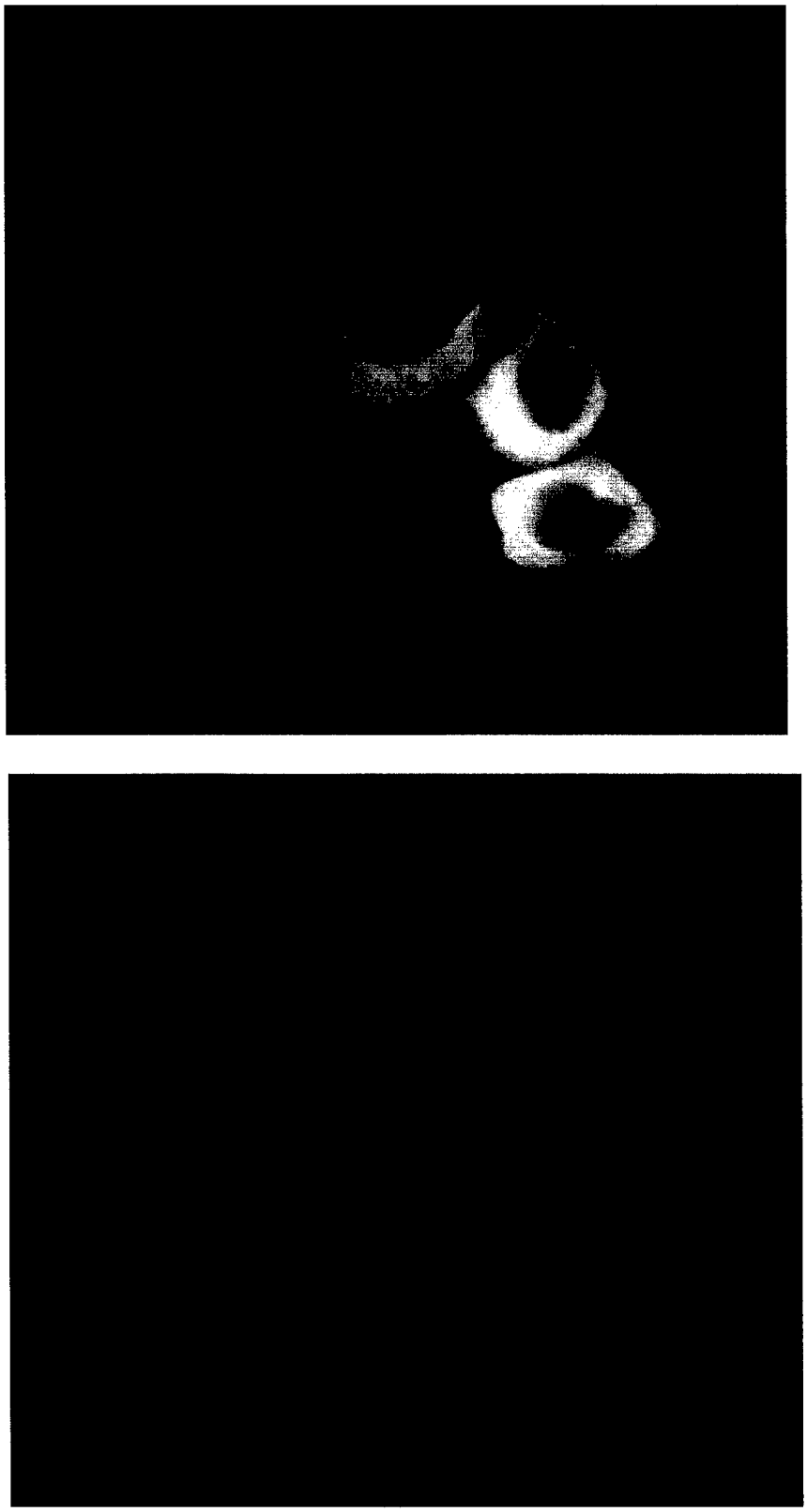

Fig. 6. Overexpression of GFP-cofactor D results in the loss of tubulin heterodimer and microtubules from HeLa cells. GFP-cofactor D is expressed in a subset of cells by transient transfection (green); the tubulin is labeled with an anti-α-tubulin monoclonal antibody, followed by rhodamine labeled second antibody (red).

KEY RESEARCH ACCOMPLISHMENTS

- Cofactor D from an adenovirus expression clone can be purified from infected cells in good yeild. This purified recombinant cofactor D is found complexed with β-tubulin.
- Loss of several amino acids from the carboxyterminus of α- or β-tubulin greatly affects their in vitro folding, and suggests that C-terminal peptides could behave as cofactor inhibitors.
- Overexpression of cofactors D and E in cultured cells is lethal, validating our hypothesis that correct cofactor functioning is vital for cancer cell survival.

REPORTABLE OUTCOMES

The data contained in this report were presented at the 14th Meeting of the European Cytoskeleton Forum held in Oeiras, Portugal, August 28th-September 2nd, 1999.

CONCLUSIONS

In the first two years of this grant we have succeeded in producing milligram quantities of recombinant tubulin-folding cofactors A, B, C, D and E. This is a vital prerequisite for the proposed search for inhibitors of these proteins. We have also shown that the proper functioning of these proteins is necessary for cancer cell survival. As yet we have not successfully found cofactor inhibitors in peptide libraries. However, proteolysis of tubulin and production of tubulin subfragments by genetic engineering show promise for the identification of inhibitors. Such inhibitors could be valuable in the treatment of breast cancer.

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